

Isolation of Human Adult Stem Cells from Muscle Biopsy for Future Treatment of Urinary Incontinence

Farzaneh Sharifiaghdas,¹ Maryam Taheri,¹ Reza Moghadasali²

Purpose: To find a suitable and cost-effective technique for isolation and culture of muscle-derived stem cells (MDSCs) obtained from muscle biopsy in large quantities.

Materials and Methods: A small muscle biopsy was taken from 10 donor rectus muscles in patients undergoing open abdominal surgery for any reason and transported on ice to the laboratory. The isolation of MDSCs was performed by two techniques; preplate and tissue explants. Initially, the isolation was carried out by preplating technique. However, enzymatic digestion of muscle biopsy in preplate technique compromised the integrity of important surface antigens of resident muscle stem cells and led to dysfunctional sorted cells. Also, many of the cells were lost in this technique and low numbers of MDSCs were yielded upon processing. Thus, we changed condition of centrifuge, but it did not affect cell numbers and their integrities. To overcome these problems, the technique was changed to tissue explants technique.

Results: During the first 4 days in explant medium culture, activated satellite cells detached, migrated, and slowly divided. The MDSCs proliferated around the native myofiber and after 2 to 3 weeks, individual muscle cells appeared elongated and fused to create large multinucleated myotubes. On immunofluorescent staining, these emerged cells were positive for desmin and Pax7 and flow cytometry analysis revealed that these cells were CD45-, CD56+, and variable in CD34.

Conclusion: We concluded that tissue explant method is a suitable and cost-effective technique for isolation and culture of MDSCs from muscle biopsy in large quantities.

Keywords: urinary incontinence, muscle cells, stem cells, tissue culture techniques

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¹Urology and Nephrology Research Center, Shahid Labbafinejad Medical Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

²Department of Stem Cells, Shahid Beheshti University of Medical Sciences, Royan Institute, Tehran, Iran

Corresponding Author:
Farzaneh Sharifiaghdas, MD
Urology and Nephrology Research Center, No.103, 9th Boustan St., Pasdaran Ave., Tehran, Iran
Tel: +98 21 22567222
Fax: +98 21 22567282
E-mail: fsharifiaghdas@yahoo.com

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INTRODUCTION

There are over 200 million people throughout the world who suffer from incontinence, a condition that is associated with social impact and a reduced quality of life.⁽¹⁾ Stress urinary incontinence (SUI) has been reported as the most common type of urinary incontinence, and cellular therapy with stem cells is a new approach to the treatment of SUI.⁽²⁾

According to previous studies,

isolation of muscle-derived stem cells (MDSCs) from muscle biopsy can be done through two techniques; preplate and tissue explant. However, enzymatic digestion of the muscle biopsy in preplate technique can compromise the integrity of important surface antigens of resident muscle stem cells, resulting in dysfunctional sorted cells, which makes the method difficult to be reproduced.⁽²⁾ Also, many of the

cells are lost in this technique and low numbers of MDSCs are yielded upon processing. Whereas the primary tissue explant technique could facilitate the proliferation of MDSCs from muscle biopsies in vitro. We demonstrated that desmin-positive CD45- CD56+ Pax7+ cells isolated by this method keep their self-renewal capacity and could then be either passaged or differentiated, resulting in the fusion of most of the mononucleated cells to produce large multinucleated myotubes.

MATERIALS AND METHODS

Stem Cell Sources

Cell Isolation: preplate and tissue explant techniques

The isolation of MDSCs was performed using previously described preplating technique.^(3,4)

A small muscle biopsy ($0.5 \times 0.5 \text{ cm}^2$) was taken from 10 donor rectus muscles in patients undergoing abdominal surgery for any reason and was collected in phosphate buffer saline medium (PBS; Gibco, Cat No.21600-051) with penicillin $0.05 \mu\text{g/mL}$ and streptomycin $0.05 \mu\text{g/mL}$ transported on ice. In laboratory, the muscle biopsy was taken out of the transport media and placed in a sterile petri dish. Then, it was minced and chopped with razor blades. The minced skeletal biopsy was subjected to a triple digest strategy. First, it was placed in a collagenase IV, solution 0.2% (Gibco, Cat No.17104-019) for 1 hour at 37°C , with agitation every 10 minutes. The solution was centrifuged at $300 \times g$ for 8 minutes, and the supernatant was discarded. Thereafter, the muscle biopsy pellet was resuspended in dispase 0.3% (Gibco, Cat No.17105-041) for 45 minutes at 37°C with agitation every 10 minutes. The solution was centrifuged at $300 \times g$ for 8 minutes and the supernatant was discarded. Finally, it was resuspended in trypsin-ethylenediaminetetraacetic acid (EDTA) 0.1% (Gibco, Cat No.25300) for 30 minutes at 37°C , with agitation every 10 minutes. The solution was centrifuged at $300 \times g$ for 8 minutes, and the supernatant was discarded. Then, the cell suspension was added to a collagen-coated flask (Nalgene, Cat No.5409) in Dulbecco's modified Eagles medium (DMEM; Gibco, Cat No.12800-

116), containing 10% fetal bovine serum (FBS; Gibco, Cat No. 10270-106), 10% horse serum, 1% penicillin-streptomycin (Gibco, Cat No. 15070), 2mM L-glutamine (L- glu) (Gibco, Cat No. 25030), and 0.5% chick embryo extract: Gibco-BRL). After 1 hour, non adherent cells in suspension were removed and transferred to a second flask for a period of 2 hours. Fresh medium was added to the first set of adherent cells (preplate1, pp1) and this procedure was continued for PP3 through PP6 at subsequent 24-hour intervals.

According to previous researches performed by this technique, six cultures of adherent cells were produced with different adhesion characteristics. Since in this study triple digest strategy led to dysfunctional sorted cells and the number of cells was very low, we changed condition of centrifuge to $1500 \times g$ for 5 minutes, but it did not affect cell numbers and properties. To resolve these problems, the technique of isolation of MDSCs was changed from preplate to tissue explant technique.^(5,6) In this technique, the minced muscle biopsies were cultured as explant in explant medium (M199, Gibco, Cat No.31150) and FBS 10% at 37°C , and 5% CO_2 .

Flow Cytometry

Flow cytometry was used at 2 to 3 weeks and 6 to 7 weeks to analyze the MDSCs population for the expression of surface proteins; CD45, CD34, and CD56. Cells were washed in PBS + FBS medium (Dulbecco's phosphate buffer saline; Gibco, Cat No.21600-051, containing 0.5% fetal bovine serum; Gibco, Cat No. 10270-106) and were fixed by paraformaldehyde 2% (Sigma, Cat No. 58H0914). Cells were again washed and permeated by Triton X-100 (Sigma, Cat No. T8787). Thereafter, the cells were washed and divided into four equal aliquots; three for combination of various monoclonal antibodies and one for control labeling as isotope IgG. Predetermined optimal amount of monoclonal antibodies (CD45, CD34, and CD56) (Miltenyi Biotec, CD45, Cat No.130-080-202, CD34, Cat No.130-081-001, and CD56, Cat No.130-090-755) were added directly to each tube for 30 minutes. These primary antibodies were conjugated with fluorescence isothiocyanate and phycoerythrin.

Finally, the cells were washed and fixed with PBS + FB+ paraformaldehyde. Then, the cells were subjected to flow cytometry using BD FACS calibur flow cytometer and CellQuest Pro software.

Immunocytochemistry

To assess the expression of proteins in relation to myogenic differentiation status, immunofluorescent analysis was performed for Pax7 and desmin. Cells were washed with PBS solution and fixed with paraformaldehyde 4%. Then, cells were washed with PBS + Tween 20 (Sigma, Cat No.P9416) solution twice, permeated with Triton X-100 solution, and washed again with PBS + Tween 20 solution twice. The cells were incubated at room temperature for 1 hour with primary antibody anti-desmin (Santa Cruz Biotechnology, Cat No.sc-14026) and anti-Pax 7 (abcam, Cat No.ab34360). After being rinsed with PBS + Tween 20 solutions, cells were incubated with secondary antibody (fluorescence isothiocyanate) (Sigma, Cat No. F1262) for 1 hour at room temperature. For visualizing, nuclei cells were washed and analyzed on an Olympus CKX 41 fluorescent microscope and Olympus DP71 camera.

RESULTS

Cell Growth

During the first 4 days in explant medium culture, activated satellite cells detached, migrated, and divided slowly. We observed that MDSCs had proliferated around the native myofiber and after

2 to 3 weeks, individual muscle cells appeared elongated and fused to create large multinucleated myotubes (Figure 1).

Flow Cytometry and Immunocytochemistry

We examined the population of MDSCs isolated by explant technique twice; at 2 to 3 weeks and 6 to 7 weeks. This flow cytometry technique was used to analyze the MDSCs population for the expression of surface proteins; CD45, CD34, and CD56. The total percentage of MDSCs at 2 to 3 weeks and 6 to 7 weeks were 4.21% and 5.91% for expressing CD45, 16.21% and 12.55% for expressing CD34, and 58.57% and 48.29% for expressing CD56, respectively. These results are demonstrated in Figure 2 by histograms. Immunochemical staining revealed that MDSCs were positive for desmin and Pax7 (Figure 3).

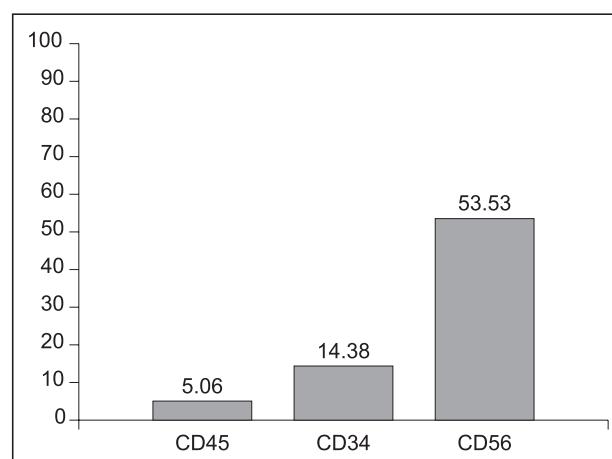


Figure 2. Mean percentage of MDSCs populations expressing cell surface proteins CD45, CD34, and CD56.

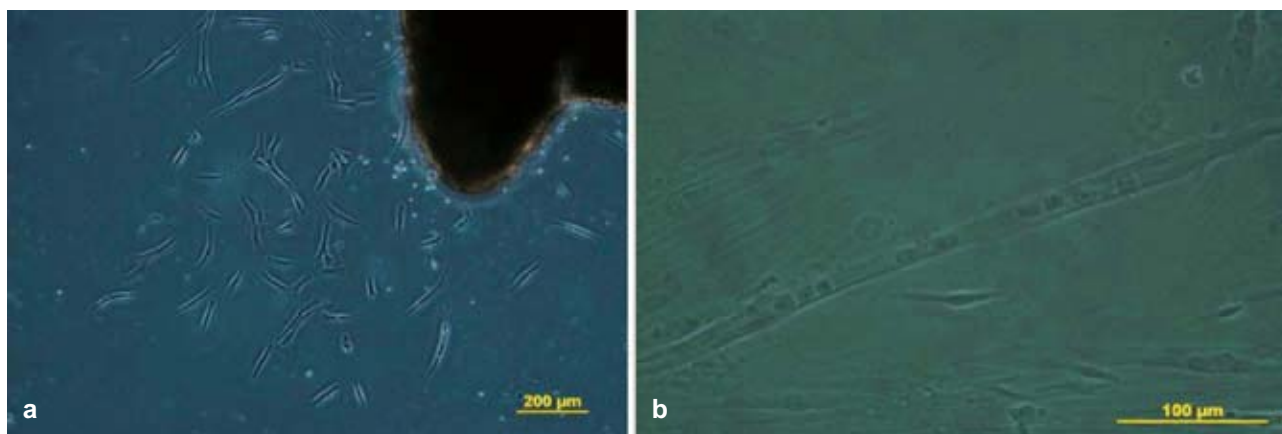


Figure 1. a) During the first 4 days in explant medium culture, activated satellite cells detached and migrated from muscle biopsy b) At 2 to 3 weeks, individual muscle cells appeared elongated and fused to create large multinucleated myotubes.

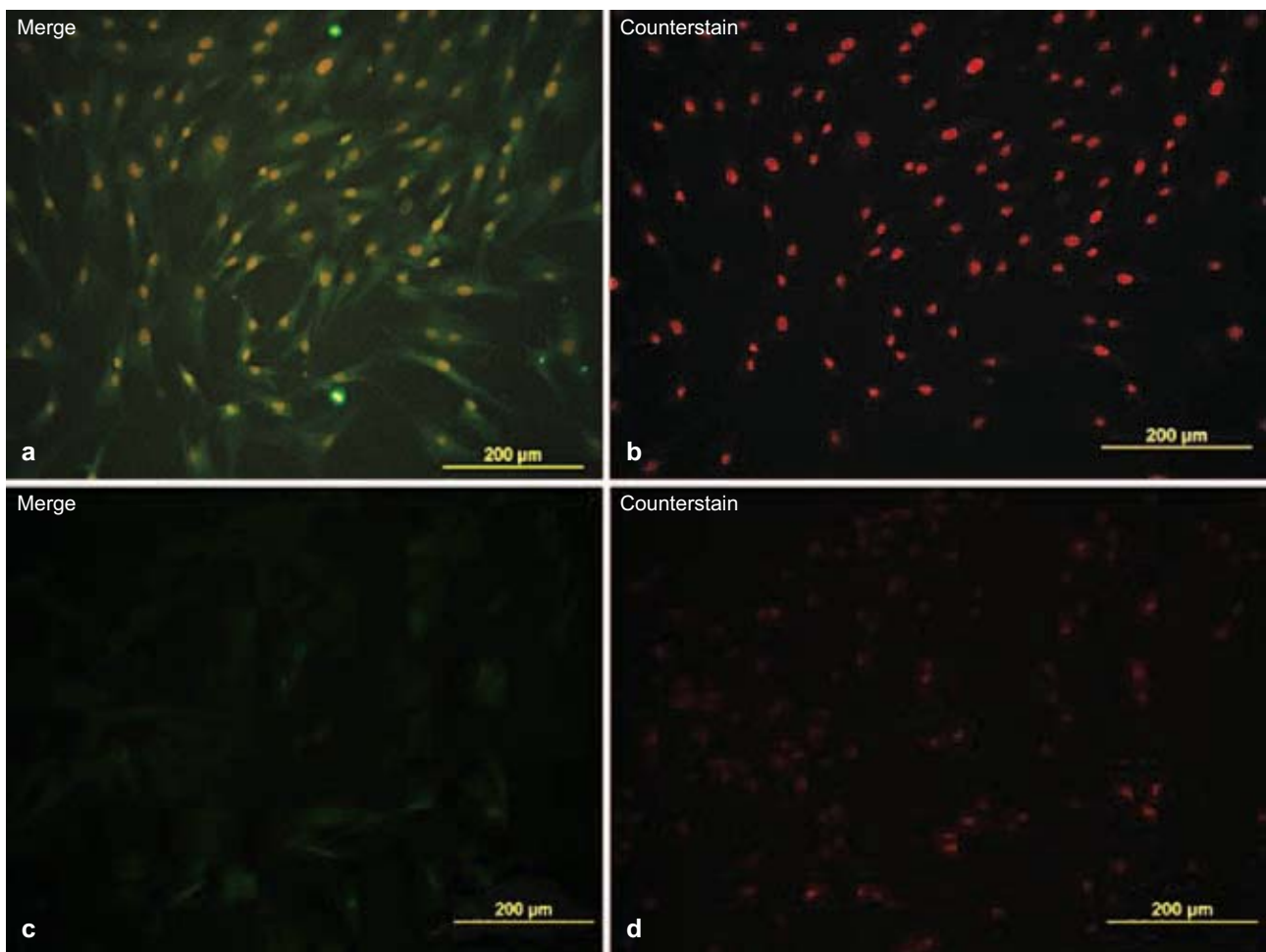


Figure 3. (a and b) Immunofluorescent staining for detection of Pax7, and (c and d) desmin in MDSCs derived from muscle biopsy by tissue culture explant technique.

DISCUSSION

Multipotent stem cells have the ability to proliferate and differentiate into different cell lines. Tissue engineering is a new science to regenerate tissues based on the use of stem cells.⁽⁷⁾ The two general types of stem cells that are potentially useful for the treatment are embryonic stem cells and adult stem cells. The practical use of embryonic stem cells is restricted because of potential regulatory problems and ethical considerations. However, there are no significant ethical issues that are related to the use of adult stem cells.⁽¹⁾

Satellite cells are a subpopulation of skeletal muscle-derived cells that are capable of self renewal and regenerating the muscle after injury, the same as the stem cells. Satellite cells, described in the classical work of Mauro,⁽⁸⁾ are situated

on the surface of the myofiber between the

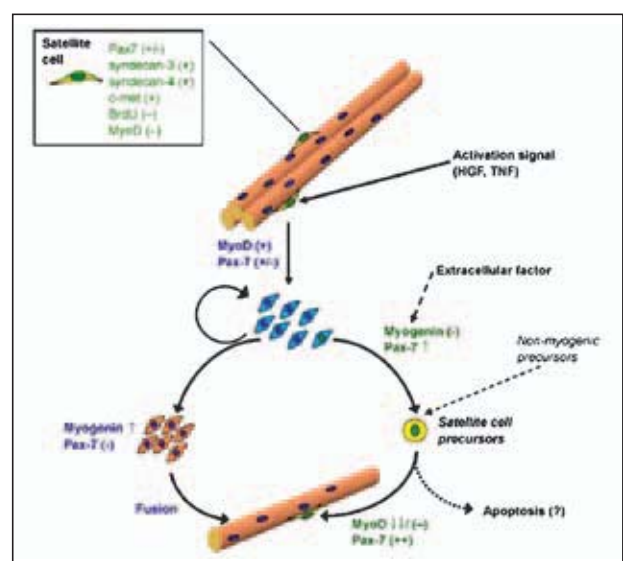


Figure 4. Working model demonstrates quiescent satellite cells which are heterogeneous for Pax7 protein.

myofiber plasmalemma and its covering basement membrane.

Various cell surface makers have been identified to purify adult stem cell population from skeletal muscle, including c-kit, sca-1, and CD34. Although nearly all muscle-derived hematopoietic progenitor cells are derived from CD45+ cells, common molecular phenotypes of MDSCs are sca-1+, CD45-, and various amounts of CD34.⁽⁹⁾ It appears that the difference with other markers might be due to culture conditions associated with different techniques or culturing protocols of different laboratories.⁽⁷⁾

In the present study, sca-1 was not evaluated since the human antibody of sca-1 was not available. Mean expression of CD45 and CD34 at 2 to 3 weeks and 5 to 6 weeks were 5.06% and 14.38%, respectively. We observed that CD45 is not a marker of MDSCs and 95% of our cells were CD45-. Peault and colleagues⁽¹⁰⁾ and Kayhanian and associates⁽¹¹⁾ proposed CD56 as another marker for myogenic cells, which had the mean expression of 53.53% in our study.

Deasy and coworkers suggested that Pax7 could be considered as a marker of the muscle satellite cell, since it is necessary and sufficient for specifying of myogenic cells.⁽⁷⁾ However, a recent study using human biopsies aimed at identifying satellite cells in vivo and revealed cells within the satellite cell position, but failed to express Pax7.⁽¹²⁾ Some researchers think that various Pax7 expressions may also result from the proliferative or cycling status of satellite cells.⁽⁷⁾

By working model that is indicated in Figure 4, Olguin and Olwin described that satellite cells are heterogeneous for Pax7 protein in quiescent state.⁽¹³⁾ Activation of satellite cells induces up regulation of MyoD. Proliferating myoblasts are heterogeneous and positive for both MyoD and Pax7. A small number of cells differentiate early, and induce myogenin and lose Pax7 expression. Another small group of cells can act as myoblast precursors. It is assumed that co-expression of both MyoD and Pax7 is necessary for myoblasts to prevent premature differentiation and keep them in a proliferative state. For the cells committed to differentiation, the myosine

program goes forward, Pax7 is down regulated and MyoD family transcription factors are up regulated. A small number of precursor cells will go opposite, up regulate Pax7 and down regulate MyoD, and form a new satellite pool.

Rouger and colleagues used M-cadherin, Pax7, and desmin expression to determine the rate of respective progression of cells toward end stage myogenic differentiation.⁽¹⁴⁾ In this study, our cells were highly positive for immunostaining of Pax7 and desmin.

CONCLUSION

We found that explant tissue culture is a suitable technique for isolation of MDSCs in large quantities and this method precludes the possible enzymatic destruction of functional stem cell markers on cell surface. Also, we demonstrated that MDSCs with desmin positive markers (CD45-, CD34+/-, CD56+, and Pax7+) have self-renewal properties and could then differentiate to myoblast, resulting in fusion of most of the mononucleated cells to produce large multinucleated myotubes in vitro. However, further studies are needed to demonstrate the applicability of these myotubes as external sphincter.

CONFLICT OF INTEREST

None declared.

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